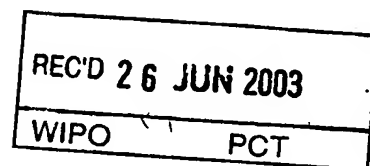


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# Kongeriget Danmark

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23 May 2003

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Bo Z. Tidemann

25 APR. 2002

## METHODS AND COMPOSITIONS FOR KILLING SPORES

Modtaget

## FIELD OF THE INVENTION

The present invention relates to enzymatic methods for killing or inactivating microbial  
5 spores.

## BACKGROUND

Spores are known to form from aerobic Bacilli, anaerobic Clostridia, selected sarcinae  
and a few actinomycetes. Spores resemble certain plant seeds in that they do not carry out any  
10 metabolic reactions. In this regard they are especially suited to withstand severe environmental  
stress and are known to survive prolonged exposures to heat, drying, radiation and toxic  
chemicals. These properties make spores especially difficult to kill in environments, like living  
tissue or objects which come in contact with living tissue, which would be adversely effected by  
extreme conditions.

15 Fungi, viruses and vegetative cells of pathogenic bacteria are sterilized within minutes at  
70 degrees Celsius; many spores are sterilized at 100 degrees Celsius. However, the spores of  
some saprophytes can survive boiling for hours. Heat is presently the most commonly used  
means to insure sterilization of spores.

A particularly difficult problem relates to microbiocidal treatment of bacterial spore-  
20 forming microorganisms of the *Bacillus cereus* group.

Microorganisms of the *Bacillus cereus* group include *Bacillus cereus*, *Bacillus mycoides*,  
*Bacillus anthracis*, and *Bacillus thuringiensis*. These microorganisms share many phenotypical  
properties, have a high level of chromosomal sequence similarity, and are known enterotoxin  
producers.

25 Although all spore-forming microorganisms are problematic for microbiocidal treatments  
because they form spores, *Bacillus cereus* is one of the most problematic because *Bacillus*  
*cereus* has been identified as possessing increased resistance to germicidal chemicals used to  
decontaminate environmental surfaces.

*Bacillus cereus* is a particularly well-established enterotoxin producer and food-borne  
30 pathogen. This organism is frequently diagnosed as a cause of gastrointestinal disorders and  
has been suggested to be the cause of several foodborne illness outbreaks. The organism is  
ubiquitous in nature, and as a consequence, is present in animal feed and fodder. Due to its  
rapid sporulating capacity, the organism easily survives in the environment and can survive  
intestinal passage in cows. The organism can contaminate raw milk via feces and soil, and  
35 *Bacillus cereus* can easily survive the pasteurization process.

The present invention provides an improved enzymatic method for killing or inactivating  
spores.

## SUMMARY

The present invention provides as a first aspect a sporocidal composition comprising a laccase or a compound exhibiting laccase activity, a source of oxygen, a source of iodide ions and an enhancing agent.

In a second aspect is provided a method of killing or inactivating spores, comprising contacting the spores with the sporocidal composition of the invention.

In a third aspect is provided a method of decontaminating a location, which has been exposed to spores, comprising contacting the spores with the composition of the invention.

In a fourth aspect is provided a container comprising the composition of the invention, wherein the components of the composition are packaged in one or more compartments or layers.

In a fifth aspect is provided a ready-to-use sporocidal formulation comprising the composition of the invention.

In embodiments, the source of iodide may be one or more salts of iodide, such as sodium iodide or potassium iodide or mixtures thereof.

In other embodiments, the sporocidal composition of the invention further comprises a surfactant.

## DETAILED DESCRIPTION

### Laccases and Compounds Exhibiting Laccase Activity

Compounds exhibiting laccase activity may be any laccase enzyme comprised by the enzyme classification EC 1.10.3.2 as set out by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB), or any fragment derived therefrom exhibiting laccase activity, or a compound exhibiting a similar activity, such as a catechol oxidase (EC 1.10.3.1), an o-aminophenol oxidase (EC 1.10.3.4), or a bilirubin oxidase (EC 1.3.3.5).

Preferred laccase enzymes and/or compounds exhibiting laccase activity are enzymes of microbial origin. The enzymes may be derived from plants, bacteria or fungi (including filamentous fungi and yeasts).

Suitable examples from fungi include a laccase derivable from a strain of *Aspergillus*, *Neurospora*, e.g., *N. crassa*, *Podospora*, *Botrytis*, *Collybia*, *Fomes*, *Lentinus*, *Pleurotus*, *Trametes*, e.g., *T. villosa* and *T. versicolor*, *Rhizoctonia*, e.g., *R. solani*, *Coprinus*, e.g., *C. cinereus*, *C. comatus*, *C. friesii*, and *C. plicatilis*, *Psathyrella*, e.g., *P. condelleana*, *Panaeolus*, e.g., *P. papilionaceus*, *Myceliophthora*, e.g., *M. thermophila*, *Schytalidium*, e.g., *S. thermophilum*, *Polyporus*, e.g., *P. pinsitus*, *Phlebia*, e.g., *P. radita* (WO 92/01046), or *Coriolus*, e.g., *C. hirsutus* (JP 2-238885).

Suitable examples from bacteria include a laccase derivable from a strain of *Bacillus*.

A laccase derived from *Coprinus*, *Myceliophthora*, *Polyporus*, *Scytalidium* or *Rhizoctonia* is preferred; in particular a laccase derived from *Coprinus cinereus*, *Myceliophthora thermophila*, *Polyporus pinsitus*, *Scytalidium thermophilum* or *Rhizoctonia solani*.

5        The laccase or the laccase related enzyme may furthermore be one which is producible by a method comprising cultivating a host cell transformed with a recombinant DNA vector which carries a DNA sequence encoding said laccase as well as DNA sequences encoding functions permitting the expression of the DNA sequence encoding the laccase, in a culture medium under conditions permitting the expression of the laccase enzyme, and recovering the  
10       laccase from the culture.

#### Determination of Laccase Activity (LACU)

Laccase activity (particularly suitable for *Polyporus* laccases) may be determined from the oxidation of syringaldazin under aerobic conditions. The violet colour produced is  
15       photometered at 530 nm. The analytical conditions are 19 mM syringaldazin, 23 mM acetate buffer, pH 5.5, 30°C, 1 min. reaction time.

1 laccase unit (LACU) is the amount of enzyme that catalyses the conversion of 1.0 mmole syringaldazin per minute at these conditions.

#### Determination of Laccase Activity (LAMU)

Laccase activity may be determined from the oxidation of syringaldazin under aerobic conditions. The violet colour produced is measured at 530 nm. The analytical conditions are 19 mM syringaldazin, 23 mM Tris/maleate buffer, pH 7.5, 30°C, 1 min. reaction time.

1 laccase unit (LAMU) is the amount of enzyme that catalyses the conversion of 1.0  
25       mmole syringaldazin per minute at these conditions.

#### Source of Oxygen

The source of oxygen required by the laccase or the compound exhibiting laccase activity may be oxygen from the atmosphere or an oxygen precursor for in situ production of oxygen.

30       Oxygen from the atmosphere will usually be present in sufficient quantity. If more O<sub>2</sub> is needed, additional oxygen may be added, e.g. as pressurized atmospheric air or as pure pressurized O<sub>2</sub>.

#### Source of Iodide ions

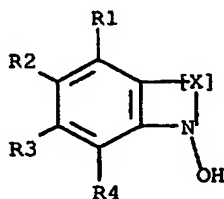
35       According to the invention the source of iodide ions needed for the reaction with the haloperoxidase may be achieved in many different ways, such as by adding one or more salts

of iodide. In a preferred embodiment the salt of iodide is sodium iodide or potassium iodide, or mixtures thereof.

The concentration of the source of iodide ions will typically correspond to a concentration of iodide ions of from 0.01 mM to 1000 mM, preferably from 0.05 mM to 500 mM, and more preferably from 0.1 mM to 100 mM.

#### Enhancing agent

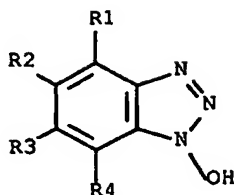
The enhancing agent may be selected from the group consisting of aliphatic, cycloaliphatic, heterocyclic or aromatic compounds containing the moiety >N-OH. In a preferred embodiment of the invention the enhancing agent is a compound of the general formula I:



wherein  $R^1$ ,  $R^2$ ,  $R^3$ ,  $R^4$  are individually selected from the group consisting of hydrogen, halogen, hydroxy, formyl, carboxy and salts and esters thereof, amino, nitro,  $C_{1-12}$ -alkyl,  $C_{1-6}$ -alkoxy, carbonyl( $C_{1-12}$ -alkyl), aryl, in particular phenyl, sulfo, aminosulfonyl, carbamoyl, phosphono, phosphonooxy, and salts and esters thereof, wherein the  $R^1$ ,  $R^2$ ,  $R^3$ ,  $R^4$  may be substituted with  $R^5$ , wherein  $R^5$  represents hydrogen, halogen, hydroxy, formyl, carboxy and salts and esters thereof, amino, nitro,  $C_{1-12}$ -alkyl,  $C_{1-6}$ -alkoxy, carbonyl( $C_{1-12}$ -alkyl), aryl, in particular phenyl, sulfo, aminosulfonyl, carbamoyl, phosphono, phosphonooxy, and salts and esters thereof; [X] represents a group selected from  $(-N=N-)$ ,  $(-N=CR^6-)_m$ ,  $(-CR^6=N-)_m$ ,  $(-CR^7=CR^8-)_m$ ,  $(-CR^6=N-NR^7-)$ ,  $(-N=N-CHR^6-)$ ,  $(-N=CR^6-NR^7-)$ ,  $(-N=CR^6-CHR^7-)$ ,  $(-CR^6=N-CHR^7-)$ ,  $(-CR^6=CR^7-NR^8-)$ , and  $(-CR^6=CR^7-CHR^8-)$ , wherein  $R^6$ ,  $R^7$ , and  $R^8$  independently of each other are selected from H, OH,  $NH_2$ ,  $COOH$ ,  $SO_3H$ ,  $C_{1-6}$ -alkyl,  $NO_2$ , CN, Cl, Br, F,  $CH_2OCH_3$ ,  $OCH_3$ , and  $COOCH_3$ ; and m is 1 or 2.

The term " $C_{1-n}$ -alkyl" wherein n can be from 2 through 12, as used herein, represent a branched or straight alkyl group having from one to the specified number of carbon atoms. Typical  $C_{1-6}$ -alkyl groups include, but are not limited to, methyl, ethyl, n-propyl, iso-propyl, butyl, iso-butyl, sec-butyl, tert-butyl, pentyl, iso-pentyl, hexyl, iso-hexyl and the like.

In a more preferred embodiment of the invention the enhancing agent is a compound of the general formula II:



wherein  $R^1$ ,  $R^2$ ,  $R^3$ ,  $R^4$  are individually selected from the group consisting of hydrogen, halogen, hydroxy, formyl, carboxy and salts and esters thereof, amino, nitro,  $C_{1-12}$ -alkyl,  $C_{1-6}$ -alkoxy, carbonyl( $C_{1-12}$ -alkyl), aryl, in particular phenyl, sulfo, aminosulfonyl, carbamoyl, phosphono, phosphonooxy, and salts and esters thereof, wherein the  $R^1$ ,  $R^2$ ,  $R^3$ ,  $R^4$  may be substituted with  $R^5$ , wherein  $R^5$  represents hydrogen, halogen, hydroxy, formyl, carboxy and salts and esters thereof, amino, nitro,  $C_{1-12}$ -alkyl,  $C_{1-6}$ -alkoxy, carbonyl( $C_{1-12}$ -alkyl), aryl, in particular phenyl, sulfo, aminosulfonyl, carbamoyl, phosphono, phosphonooxy, and salts and esters thereof.

The enhancing agent may also be a salt or an ester of formula I or II.

Further preferred enhancing agents are oxoderivatives and N-hydroxy derivatives of heterocyclic compounds and oximes of oxo- and formyl-derivatives of heterocyclic compounds, said heterocyclic compounds including five-membered nitrogen-containing heterocycles, in particular pyrrol, pyrazole and imidazole and their hydrogenated counterparts (e.g. pyrrolidine) as well as triazoles, such as 1,2,4-triazole; six-membered nitrogen-containing heterocycles, in particular mono-, di- and triazinanes (such as piperidine and piperazine), morpholine and their unsaturated counterparts (e.g. pyridine and pyrimidine); and condensed heterocycles containing the above heterocycles as substructures, e.g. indole, benzothiazole, quinoline and benzoazepine.

Examples of preferred enhancing agent from these classes of compounds are pyridine aldoximes; N-hydroxypyrrolidinediones such as N-hydroxysuccinimide and N-hydroxyphthalimide; 3,4-dihydro-3-hydroxybenzo[1,2,3]triazine-4-one; formaldoxime trimer (N,N',N''-trihydroxy-1,3,5-triazinane); and violuric acid (1,3-diazinane-2,4,5,6-tetrone-5-oxime).

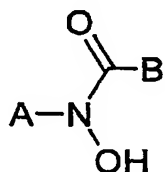
Still further enhancing agents which may be applied in the invention include oximes of oxo- and formyl-derivatives of aromatic compounds, such as benzoquinone dioxime and salicylaldoxime (2-hydroxybenzaldehyde oxime), and N-hydroxyamides and N-hydroxyanilides, such as N-hydroxyacetanilide.

Preferred enhancing agents are selected from the group consisting of 1-hydroxybenzotriazole; 1-hydroxybenzotriazole hydrate; 1-hydroxybenzotriazole sodium salt; 1-hydroxybenzotriazole potassium salt; 1-hydroxybenzotriazole lithium salt; 1-hydroxybenzotriazole ammonium salt; 1-hydroxybenzotriazole calcium salt; 1-hydroxybenzotriazole magnesium salt; and 1-hydroxybenzotriazole-6-sulphonic acid.

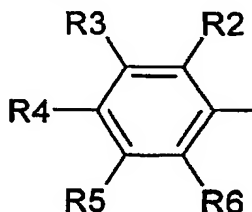
A particularly preferred enhancing agent is 1-hydroxybenzotriazole.

All the specifications of N-hydroxy compounds above are understood to include tautomeric forms such as N-oxides whenever relevant.

Another preferred group of enhancing agents comprises a -CO-NOH- group and has the  
5 general formula III:



in which A is:



and B is the same as A; or B is H or C<sub>1-12</sub>-alkyl, said alkyl may contain hydroxy, ester or ether  
10 groups (e.g. wherein the ether oxygen is directly attached to A-N(OH)C=O-, thus including N-hydroxy carbamic acid ester derivatives), and R2, R3, R4, R5 and R6 independently of each other are H, OH, NH<sub>2</sub>, COOH, SO<sub>3</sub>H, C<sub>1-8</sub>-alkyl, acyl, NO<sub>2</sub>, CN, Cl, Br, F, CF<sub>3</sub>, NOH-CO-phenyl, CO-NOH-phenyl, C<sub>1-6</sub>-CO-NOH-A, CO-NOH-A, COR12, phenyl-CO-NOH-A, OR7, NR8R9, COOR10, or NOH-CO-R11, wherein R7, R8, R9, R10, R11 and R12 are C<sub>1-12</sub>-alkyl or acyl.

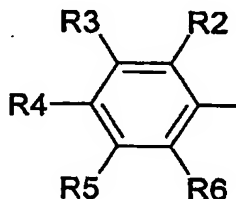
15 R2, R3, R4, R5 and R6 of A are preferably H, OH, NH<sub>2</sub>, COOH, SO<sub>3</sub>H, C<sub>1-3</sub>-alkyl, acyl, NO<sub>2</sub>, CN, Cl, Br, F, CF<sub>3</sub>, NOH-CO-phenyl, CO-NOH-phenyl, COR12, OR7, NR8R9, COOR10, or NOH-CO-R11, wherein R7, R8 and R9 are C<sub>1-3</sub>-alkyl or acyl, and R10, R11 and R12 are C<sub>1-3</sub>-alkyl; more preferably R2, R3, R4, R5 and R6 of A are H, OH, NH<sub>2</sub>, COOH, SO<sub>3</sub>H, CH<sub>3</sub>, acyl, NO<sub>2</sub>, CN, Cl, Br, F, CF<sub>3</sub>, CO-NOH-phenyl, COCH<sub>3</sub>, OR7, NR8R9, or COOCH<sub>3</sub>, wherein R7, R8  
20 and R9 are CH<sub>3</sub> or COCH<sub>3</sub>; even more preferably R2, R3, R4, R5 and R6 of A are H, OH, COOH, SO<sub>3</sub>H, CH<sub>3</sub>, acyl, NO<sub>2</sub>, CN, Cl, Br, F, CO-NOH-phenyl, OCH<sub>3</sub>, COCH<sub>3</sub>, or COOCH<sub>3</sub>; and in particular R2, R3, R4, R5 and R6 of A are H, OH, COOH, SO<sub>3</sub>H, CH<sub>3</sub>, NO<sub>2</sub>, CN, Cl, Br, CO-NOH-phenyl, or OCH<sub>3</sub>.

R2, R3, R4, R5 and R6 of B are preferably H, OH, NH<sub>2</sub>, COOH, SO<sub>3</sub>H, C<sub>1-3</sub>-alkyl, acyl,  
25 NO<sub>2</sub>, CN, Cl, Br, F, CF<sub>3</sub>, NOH-CO-phenyl, CO-NOH-phenyl, COR12, OR7, NR8R9, COOR10, or NOH-CO-R11, wherein R7, R8 and R9 are C<sub>1-3</sub>-alkyl or acyl, and R10, R11 and R12 are C<sub>1-3</sub>-alkyl; more preferably R2, R3, R4, R5 and R6 of B are H, OH, NH<sub>2</sub>, COOH, SO<sub>3</sub>H, CH<sub>3</sub>, acyl, NO<sub>2</sub>, CN, Cl, Br, F, CF<sub>3</sub>, CO-NOH-phenyl, COCH<sub>3</sub>, OR7, NR8R9, or COOCH<sub>3</sub>, wherein R7, R8 and R9 are CH<sub>3</sub> or COCH<sub>3</sub>; even more preferably R2, R3, R4, R5 and R6 of B are H, OH,

COOH, SO<sub>3</sub>H, CH<sub>3</sub>, acyl, NO<sub>2</sub>, CN, Cl, Br, F, CO-NOH-phenyl, OCH<sub>3</sub>, COCH<sub>3</sub>, or COOCH<sub>3</sub>; and in particular R<sub>2</sub>, R<sub>3</sub>, R<sub>4</sub>, R<sub>5</sub> and R<sub>6</sub> of B are H, OH, COOH, SO<sub>3</sub>H, CH<sub>3</sub>, NO<sub>2</sub>, CN, Cl, Br, CO-NOH-phenyl, or OCH<sub>3</sub>.

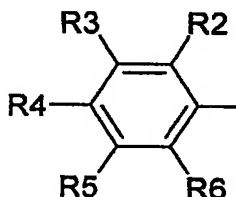
- B is preferably H or C<sub>1-3</sub>-alkyl, said alkyl may contain hydroxy, ester or ether groups; preferably said alkyl may contain ester or ether groups; more preferably said alkyl may contain ether groups.

In an embodiment, A and B independently of each other are:



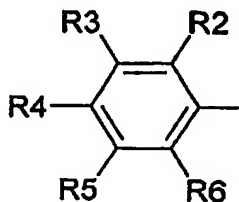
- or B is H or C<sub>1-3</sub>-alkyl, said alkyl may contain hydroxy, ester or ether groups (e.g. wherein the ether oxygen is directly attached to A-N(OH)C=O-, thus including N-hydroxy carbamic acid ester derivatives), and R<sub>2</sub>, R<sub>3</sub>, R<sub>4</sub>, R<sub>5</sub> and R<sub>6</sub> independently of each other are H, OH, NH<sub>2</sub>, COOH, SO<sub>3</sub>H, C<sub>1-3</sub>-alkyl, acyl, NO<sub>2</sub>, CN, Cl, Br, F, CF<sub>3</sub>, NOH-CO-phenyl, CO-NOH-phenyl, COR<sub>12</sub>, OR<sub>7</sub>, NR<sub>8</sub>R<sub>9</sub>, COOR<sub>10</sub>, or NOH-CO-R<sub>11</sub>, wherein R<sub>7</sub>, R<sub>8</sub> and R<sub>9</sub> are C<sub>1-3</sub>-alkyl or acyl, and R<sub>10</sub>, R<sub>11</sub> and R<sub>12</sub> are C<sub>1-3</sub>-alkyl.

- In another embodiment, A and B independently of each other are:



- or B is H or C<sub>1-3</sub>-alkyl, said alkyl may contain hydroxy or ether groups (e.g. wherein the ether oxygen is directly attached to A-N(OH)C=O-, thus including N-hydroxy carbamic acid ester derivatives), and R<sub>2</sub>, R<sub>3</sub>, R<sub>4</sub>, R<sub>5</sub> and R<sub>6</sub> independently of each other are H, OH, NH<sub>2</sub>, COOH, SO<sub>3</sub>H, CH<sub>3</sub>, acyl, NO<sub>2</sub>, CN, Cl, Br, F, CF<sub>3</sub>, CO-NOH-phenyl, COCH<sub>3</sub>, OR<sub>7</sub>, NR<sub>8</sub>R<sub>9</sub>, or COOCH<sub>3</sub>, wherein R<sub>7</sub>, R<sub>8</sub> and R<sub>9</sub> are CH<sub>3</sub> or COCH<sub>3</sub>.

In another embodiment, A and B independently of each other are:

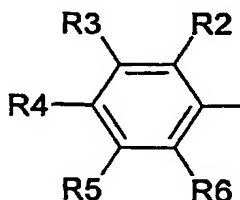


- or B is H or C<sub>1-3</sub>-alkyl, said alkyl may contain hydroxy or ether groups (e.g. wherein the ether oxygen is directly attached to A-N(OH)C=O-, thus including N-hydroxy carbamic acid ester



derivatives), and R2, R3, R4, R5 and R6 independently of each other are H, OH, COOH, SO<sub>3</sub>H, CH<sub>3</sub>, acyl, NO<sub>2</sub>, CN, Cl, Br, F, CO-NOH-phenyl, OCH<sub>3</sub>, COCH<sub>3</sub>, or COOCH<sub>3</sub>.

In another embodiment, A and B independently of each other are:



- 5 or B is C<sub>1-3</sub>-alkyl, said alkyl may contain ether groups (e.g. wherein the ether oxygen is directly attached to A-N(OH)C=O-, thus including N-hydroxy carbamic acid ester derivatives), and R2, R3, R4, R5 and R6 independently of each other are H, OH, COOH, SO<sub>3</sub>H, CH<sub>3</sub>, NO<sub>2</sub>, CN, Cl, Br, CO-NOH-phenyl, or OCH<sub>3</sub>.

10 The terms "C<sub>1-n</sub>-alkyl" wherein n can be from 2 through 12, as used herein, represent a branched or straight alkyl group having from one to the specified number of carbon atoms. Typical C<sub>1-6</sub>-alkyl groups include, but are not limited to, methyl, ethyl, n-propyl, iso-propyl, butyl, iso-butyl, sec-butyl, tert-butyl, pentyl, iso-pentyl, hexyl, iso-hexyl and the like.

15 The term "acyl" as used herein refers to a monovalent substituent comprising a C<sub>1-6</sub>-alkyl group linked through a carbonyl group; such as e.g. acetyl, propionyl, butyryl, isobutyryl, pivaloyl, valeryl, and the like.

20 In an embodiment at least one of the substituents R2, R3, R4, R5 and R6 of A are H, preferably at least two of the substituents R2, R3, R4, R5 and R6 of A are H, more preferably at least three of the substituents R2, R3, R4, R5 and R6 of A are H, most preferably at least four of the substituents R2, R3, R4, R5 and R6 of A are H, in particular all of R2, R3, R4, R5 and R6 of A are H.

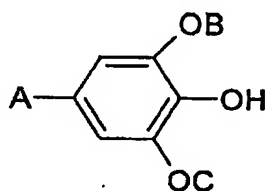
25 In another embodiment at least one of the substituents R2, R3, R4, R5 and R6 of B are H, preferably at least two of the substituents R2, R3, R4, R5 and R6 of B are H, more preferably at least three of the substituents R2, R3, R4, R5 and R6 of B are H, most preferably at least four of the substituents R2, R3, R4, R5 and R6 of B are H, in particular all of R2, R3, R4, R5 and R6 of B are H.

In particular embodiments according to the invention the enhancing agent is selected from the group consisting of

30 4-nitrobenzoic acid-N-hydroxyanilide;  
4-methoxybenzoic acid-N-hydroxyanilide;  
N,N'-dihydroxy-N,N'-diphenylterephthalamide;  
decanoic acid-N-hydroxyanilide;  
N-hydroxy-4-cyanoacetanilide;  
N-hydroxy-4-acetylacetanilide;

- N-hydroxy-4-hydroxyacetanilide;  
 N-hydroxy-3-(N'-hydroxyacetamide)acetanilide;  
 4-cyanobenzoic acid-N-hydroxyanilide;  
 N-hydroxy-4-nitroacetanilide;  
 5 N-hydroxyacetanilide;  
 N-hydroxy-N-phenyl-carbamic acid isopropyl ester;  
 N-hydroxy-N-phenyl-carbamic acid methyl ester;  
 N-hydroxy-N-phenyl-carbamic acid phenyl ester;  
 N-hydroxy-N-phenyl-carbamic acid ethyl ester; and  
 10 N-hydroxy-N-(4-cyanophenyl)-carbamic acid methyl ester.

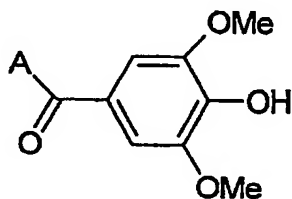
Another group of preferred enhancing agents is phenolic compounds (alkylsyringates) of the general formula IV:



- 15 wherein the letter A in said formula denotes be a group such as -D, -CH=CH-D, -CH=CH-CH=CH-D, -CH=N-D, -N=N-D, or -N=CH-D, in which D is selected from the group consisting of -CO-E, -SO<sub>2</sub>-E, -N-XY, and -N<sup>+</sup>-XYZ, in which E may be -H, -OH, -R, or -OR, and X and Y and Z may be identical or different and selected from -H and -R; R being a C<sub>1</sub>-C<sub>16</sub> alkyl, preferably a C<sub>1</sub>-C<sub>8</sub> alkyl, which alkyl may be saturated or unsaturated, branched or unbranched and  
 20 optionally substituted with a carboxy, sulpho or amino group; and B and C may be the same or different and selected from C<sub>m</sub>H<sub>2m+1</sub>, where m = 1, 2, 3, 4 or 5.

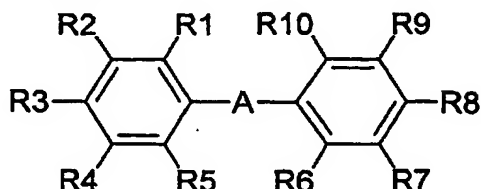
In the above mentioned general formula IV, A may be placed meta to the hydroxy group instead of being placed in the para-position as shown.

- 25 In particular embodiments of the invention the enhancing agent is selected from the group having the general formula V:



in which A is a group such as -H, -OH, -CH<sub>3</sub>, -OCH<sub>3</sub>, -O(CH<sub>2</sub>)<sub>n</sub>CH<sub>3</sub>, where n = 1, 2, 3, 4, 5, 6, 7 or 8.

Yet another group of preferred enhancing agents are the compounds as described in general formula VI:



in which general formula A represents a single bond, or one of the following groups: (-CH<sub>2</sub>-), (-CH=CH-), (-NR<sub>11</sub>-), (-CH=N-), (-N=N-), (-CH=N-N=CH-), or (>C=O);

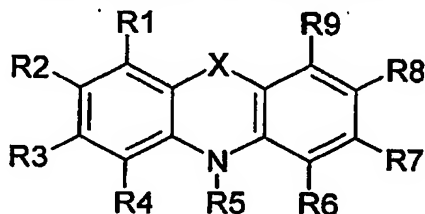
and in which general formula the substituent groups R<sub>1</sub>-R<sub>11</sub>, which may be identical or different, independently represents any of the following radicals: hydrogen, halogen, hydroxy, formyl, acetyl, carboxy and esters and salts hereof, carbamoyl, sulfo and esters and salts hereof, sulfamoyl, methoxy, nitro, amino, phenyl, C<sub>1-8</sub>-alkyl;

which carbamoyl, sulfamoyl, phenyl, and amino groups may furthermore be unsubstituted or substituted once or twice with a substituent group R<sub>12</sub>; and which C<sub>1-8</sub>-alkyl group may be saturated or unsaturated, branched or unbranched, and may furthermore be unsubstituted or substituted with one or more substituent groups R<sub>12</sub>;

which substituent group R<sub>12</sub> represents any of the following radicals: hydrogen, halogen, hydroxy, formyl, acetyl, carboxy and esters and salts hereof, carbamoyl, sulfo and esters and salts hereof, sulfamoyl, methoxy, nitro, amino, phenyl, or C<sub>1-8</sub>-alkyl; which carbamoyl, sulfamoyl, and amino groups may furthermore be unsubstituted or substituted once or twice with hydroxy or methyl.

and in which general formula R<sub>5</sub> and R<sub>6</sub> may together form a group -B-, in which B represents a single bond, one of the following groups (-CH<sub>2</sub>-), (-CH=CH-), (-CH=N-); or B represents sulfur, or oxygen.

In particular embodiments of the invention the enhancing agent is selected from the group having the general formula VII:



in which general formula X represents a single bond, oxygen, or sulphur;

and in which general formula the substituent groups R<sub>1</sub>-R<sub>9</sub>, which may be identical or different, independently represents any of the following radicals: hydrogen, halogen, hydroxy, formyl, acetyl, carboxy and esters and salts hereof, carbamoyl, sulfo and esters and salts hereof, sulfamoyl, methoxy, nitro, amino, phenyl, C<sub>1-8</sub>-alkyl;

which carbamoyl, sulfamoyl, phenyl, and amino groups may furthermore be unsubstituted or substituted once or twice with a substituent group R10; and which C<sub>1-8</sub>-alkyl group may be saturated or unsaturated, branched or unbranched, and may furthermore be unsubstituted or substituted with one or more substituent groups R10;

5 which substituent group R10 represents any of the following radicals: hydrogen, halogen, hydroxy, formyl, acetyl, carboxy and esters and salts hereof, carbamoyl, sulfo and esters and salts hereof, sulfamoyl, methoxy, nitro, amino, phenyl, or C<sub>1-8</sub>-alkyl; which carbamoyl, sulfamoyl, and amino groups may furthermore be unsubstituted or substituted once or twice with hydroxy or methyl.

10 According to the invention, the enhancing agent may be present in the composition in a concentration in the range of from 0.01 mM to 1000 mM, preferably in the range of from 0.05 mM to 500 mM, more preferably in the range of from 0.1 mM to 100 mM, and most preferably in the range of from 0.1 mM to 50 mM.

### 15 Spores

The spores which are contacted with a haloperoxidase, a source of hydrogen peroxide and a source of iodide ions in the method of the invention comprise all kinds of spores.

In an embodiment the spores are endospores, such as all *Clostridium* sp. spores,  
20 *Brevibacillus* sp. spores and *Bacillus* sp. spores, e.g. spores from *Bacillus anthracis*, *Bacillus cereus*, *Bacillus mycoides*, *Bacillus thuringiensis*, *Bacillus subtilis*, *Bacillus putida*, and *Bacillus pumila*.

In another embodiment the spores are exospores, such as *Actinomycetales* spores, e.g. spores from *Actinomyces* sp., *Streptomyces* sp., *Thermoactinomyces* sp., *Saccharomonospora*  
25 sp., and *Saccharopylospora* sp.

In another embodiment the spores are bacterial spores. Examples of bacterial spores include, but are not limited to, all *Clostridium* sp. spores and *Bacillus* sp. spores as mentioned above.

In yet another embodiment the spores are fungal spores. Examples of fungal spores  
30 include (in addition to those mentioned above), but are not limited to, conidiospores, such as spores from *Aspergillus* sp., and *Penicillium* sp.

### Surfactants

35 The surfactants suitable for being incorporated in the sporocidal composition may be non-ionic (including semi-polar), anionic, cationic and/or zwitterionic. The surfactants are preferably anionic or non-ionic. The surfactants are typically present in the sporocidal composition at a concentration of from 0.01% to 10% by weight.

When included therein, the sporocidal composition will usually contain from about 0.01% to about 10%, preferably about 0.05% to about 5%, and more preferably about 0.1% to about 1% by weight of an anionic surfactant, such as linear alkylbenzenesulfonate, alpha-olefinsulfonate, alkyl sulfate (fatty alcohol sulfate), alcohol ethoxysulfate, secondary alkanesulfonate, alpha-sulfo fatty acid methyl ester, alkyl- or alkenylsuccinic acid or soap.

When included therein the sporocidal composition will usually contain from about 0.01% to about 10%, preferably about 0.05% to about 5%, and more preferably about 0.1% to about 1% by weight of a non-ionic surfactant, such as alcohol ethoxylate, nonylphenol ethoxylate, alkylpolyglycoside, alkyl dimethylamineoxide, ethoxylated fatty acid monoethanolamide, fatty acid monoethanolamide, polyhydroxy alkyl fatty acid amide, or N-acyl N-alkyl derivatives of glucosamine ("glucamides").

### Compositions

The present invention provides a composition comprising a laccase or a compound exhibiting laccase activity, a source of oxygen, a source of iodide ions and an enhancing agent.

The laccase or the compound exhibiting laccase activity, the source of iodide ions and the enhancing agent may be formulated as a liquid (e.g. aqueous), a solid, a gel, a paste or a dry product formulation. The dry product formulation may subsequently be re-hydrated to form an active liquid or semi-liquid formulation usable in the method of the invention.

When the laccase or the compound exhibiting laccase activity, the source of iodide ions and the enhancing agent are formulated as a dry formulation, the components may be mixed, arranged in discrete layers or packaged separately.

When formulated as a solid, all components may be mixed together, e.g., as a powder, a granulate or a gelled product.

When other than dry form compositions are used and even in that case, it is preferred to use a two-part formulation system having the enzyme(s) separate from the rest of the composition.

The composition of the invention may further comprise auxiliary agents such as wetting agents, thickening agents, buffer, stabilisers, perfume, colourants, fillers and the like.

Useful wetting agents are surfactants, i.e. non-ionic, anionic, amphoteric or zwitterionic surfactants. Surfactants are further described above.

The composition of the invention may be a concentrated product or a ready-to-use product. In use, the concentrated product is typically diluted with water to provide a medium having an effective sporocidal activity, applied to the object to be cleaned or disinfected, and allowed to react with the spores present.

The pH of an aqueous solution of the composition is in the range of from pH 2 to 11, preferably in the range of from pH 3 to 10.5, more preferably in the range of from pH 4 to 10, most preferably in the range of from pH 5 to 9, and in particular in the range of from pH 6 to 8.

## 5 Methods and Uses

The present invention provides an enzymatic method for killing or inactivating spores, comprising contacting the spores with a laccase or a compound exhibiting laccase activity, a source of oxygen, a source of iodide ions and an enhancing agent.

10 In the context of the present invention the term "killing or inactivating spores" is intended to mean that at least 99% of the spores are not capable of transforming (germinating) into vegetative cells. Preferably 99.9% (more preferably 99.99% and most preferably 99.999%) of the spores are not capable of transforming into vegetative cells.

15 The spores may be contacted by the composition of the invention at a temperature between 0 and 90 degrees Celsius, preferably between 5 and 80 degrees Celsius, more preferably between 10 and 70 degrees Celsius, even more preferably between 15 and 60 degrees Celsius, most preferably between 18 and 50 degrees Celsius, and in particular between 20 and 40 degrees Celsius.

20 The composition of the invention is suitable for killing or inactivating spores in a variety of environments. The composition of the invention may desirably be used in any environment to reduce spore contamination, such as the health-care industry (e.g. animal hospitals, human hospitals, animal clinics, human clinics, nursing homes, day-care facilities for children or senior citizens, etc.), the food industry (e.g. restaurants, food-processing plants, food-storage plants, grocery stores, etc.), the hospitality industry (e.g. hotels, motels, resorts, cruise ships, etc.), the education industry (e.g. schools and universities), etc.

25 The composition of the invention may desirably be used in any environment to reduce spore contamination, such as general-premise surfaces (e.g. floors, walls, ceilings, exterior of furniture, etc.), specific-equipment surfaces (e.g. hard surfaces, manufacturing equipment, processing equipment, etc.), textiles (e.g. cottons, wools, silks, synthetic fabrics such as polyesters, polyolefins, and acrylics, fiber blends such as cottonpolyester, etc.), wood and  
30 cellulose-based systems (e.g. paper), soil, animal carcasses (e.g. hide, meat, hair, feathers, etc.), foodstuffs (e.g. fruits, vegetables, nuts, meats, etc.), and water.

In one embodiment, the method of the invention is directed to sporocidal treatment of textiles. Spores of the *Bacillus cereus* group have been identified as the predominant postlaundering contaminant of textiles. Thus, the treatment of textiles with a composition of the  
35 invention is particularly useful for sporocidal activity against the contaminants of textiles.

Examples of textiles that can be treated with the composition of the invention include, but are not limited to, personal items (e.g. shirts, pants, stockings, undergarments, etc.),

institutional items (e.g. towels, lab coats, gowns, aprons, etc.), hospitality items (e.g. towels, napkins, tablecloths, etc.).

5 A sporocidal treatment of textiles with a composition of the invention may include contacting a textile with a composition of the invention. This contacting can occur prior to laundering the textile. Alternatively, this contacting can occur during laundering of the textile to provide sporocidal activity and optionally provide cleansing activity to remove or reduce soils, stains, etc. from the textile.

10 The spores which are contacted by the composition of the invention may be situated on any surface including, but not limited to, a surface of a process equipment used in e.g. a dairy, a chemical or pharmaceutical process plant, a piece of laboratory equipment, a water sanitation system, an oil processing plant, a paper pulp processing plant, a water treatment plant, or a cooling tower. The composition of the invention should be used in an amount, which is effective for killing or inactivating the spores on the surface in question.

15 The spores may be contacted with the composition of the invention by submerging the spores in an aqueous formulation of the composition (e.g. a laundering process), by spraying the composition onto the spores, by applying the composition to the spores by means of a cloth, or by any other method recognized by the skilled person. Any method of applying the composition of the invention to the spores, which results in killing or inactivating the spores, is an acceptable method of application.

20 The method of the invention is also useful for decontamination of locations which have been exposed to spores (e.g. pathogenic spores), such as biological warfare agents, e.g. spores of *Bacillus anthracis* capable of causing anthrax. Such locations include, but are not limited to, clothings (such as army clothings), inner and outer parts of vehicles, inner and outer parts of buildings, any kind of army facility, and any kind of environment mentioned above.

25 The present invention is further described by the following examples which should not be construed as limiting the scope of the invention.

## EXAMPLES

30 Chemicals used as buffers and substrates were commercial products of at least reagent grade.

### EXAMPLE 1

#### Production of Spores

35 Streak a Tryptose Blod Agar Base (TBAB) plate from a fresh culture of *Bacillus globigii* or *B. thuringiensis* (*Bacillus thuringiensis* type strain ATCC10792). Incubate the culture overnight at 30 degrees Celsius.

Suspend a loopfull of pure *Bacillus* from the TBAB plate and suspend the cells in 2 ml of sterile water. Inoculate 2xSG plates with 100 µl of the cell suspension on each. The composition of 2xSG is as follows: 16 g/L Difco Bacto Nutrient Broth, 0.5 g/L MgSO<sub>4</sub> x 7H<sub>2</sub>O, 2.0 g/L KCl, 1.0 ml/100 ml of 10% glucose, 0.1 ml/100 ml of 1 M Ca(NO<sub>3</sub>)<sub>2</sub>, 0.1 ml/100 ml of 0.1 M MnSO<sub>4</sub>, 10 µl/100 ml of 0.01M FeSO<sub>4</sub>, and 1% Difco Bacto Agar.

Incubate plates for 48-72 hrs. at 30 degrees Celsius. Check for sporulation with phase-contrast microscopy. Spores are phase-bright.

When sporulation efficiency is close to 100%, harvest the cell lawn with water and suspend the cells by intensive vortexing. Collect cells by centrifugation for 5-10 minutes at 6000G at 4 degrees Celsius. Wash cells 3 x with ice cold water. The pellet contains vegetative cells and spores.

Apply a step-density gradient for separation of the spores from the vegetative cells. Prepare for each washed pellet a centrifuge tube containing 30 ml 43% Urographin®. Prepare 3 ml of cell spore mixture in Urographin so that the final Urographin concentration is 20%. Gently load the 20% Urographin cell/spore mixture onto the top layer of the 43% Urographin. Centrifuge at 10000G at room temperature for 30 minutes. Gently remove supernatant. Suspend the pure spore pellet in 1 ml ice-cold water and transfer to a microfuge tube. Centrifuge at maximum speed for 1-2 min at 4 degrees Celsius, wash pellet in ice-cold water 2 more times.

Check purity and number of spores/ml by phase contrast microscopy and a haemocytometer. Store spores suspended in water at -20 degrees Celsius.

## EXAMPLE 2

### Killing of Spores

The following reagents were prepared:

DMG buffer (DiMethylGlutamic acid, Sigma D4379), 50 mM, pH adjusted to 6.5 with NaOH;

Spores were re-suspended in DMG buffer to a density of  $2 \times 10^8$  spores per ml;

*Myceliophthora thermophila* laccase was diluted to 200 microgram per ml in DMG buffer;

200 mM Potassium iodide (KI) solution in DMG buffer;

1 mM Methylsyringate (methyl 3,5-dimethoxy-4-hydroxybenzoate, Sigma S40,944-8) solution in DMG buffer;

3 mM MTT (3-(4,5-Dimethylthiazol-yl)-2,5-diphenyltetrazolium bromide, Sigma M2128) solution in water.

TBB growth medium:

10 g/l Tryptose,



3 g/l Beef Extract,  
5 g/l NaCl,  
water ad 1000 ml  
final pH 7.2 +/- 0.2.

5

Spore suspension was pipetted into the wells in row A of a microtiter plate. The other reagents were added as indicated in table 1 below. The reaction was initiated by the addition of laccase solution.

10 Table 1.

Wells	DMG buffer (microliter)	Spores (microliter)	Laccase (microliter)	KI (microliter)	Methylsyringate (microliter)
A1-A2	115	50	20	5	10
A3-A4	120	50	20	0	10
A5-A6	125	50	20	5	0
A7-A8	145	50	0	5	0
A9-A10	130	50	20	0	0
A11-A12	150	50	0	0	0

15

The microtiter plate was incubated at room temperature (24 degrees Celsius) for 1 hour. 180 microliter TBB growth medium was added to all wells in rows B to H of the microtiter plate. Serial 10 fold dilutions were made by pipetting 20 microliter from row A to row B, and then from row B to row C, and then from row C to row D, and so on until row H.

20

The microtiter plate was incubated at 30 degrees Celsius for 12-24 hours to allow spores to germinate and grow. Growth was evaluated by a microplate reader and visually by "developing the growth" by addition of 5 microliter 3 mM MTT to each well. Formation of purple formazans reveals bacterial growth and thus the degree of spore inactivation.

Table 2. Results of evaluation of growth.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	-	-	+	+	+	+	+	+	+	+	+	+
C	-	-	+	+	+	+	+	+	+	+	+	+
D	-	-	+	+	+	+	+	+	+	+	+	+
E	-	-	+	+	+	+	+	+	+	+	+	+
F	-	-	+	+	+	+	+	+	+	+	+	+
G	-	-	+	-	+	+	+	+	+	+	+	+
H	-	-	-	-	-	+	+	+	+	-	+	-

The results in Table 2 shows that only the formulation added to wells A1-A2 including both  
5 laccase, potassium iodide and enhancing agent (methylsyningate) is capable of inactivating the  
spores.

**CLAIMS**

1. A sporocidal composition comprising a laccase or a compound exhibiting laccase activity, a source of oxygen, a source of iodide ions and an enhancing agent.
- 5 2. The composition of claim 1, wherein the source of iodide ions is one or more salts of iodide.
3. The composition of claim 1, which further comprises a surfactant.
- 10 4. An enzymatic method of killing or inactivating spores, comprising contacting the spores with a laccase or a compound exhibiting laccase activity, a source of oxygen, a source of iodide ions and an enhancing agent.
- 15 5. The method of claim 4, wherein the source of iodide ions is one or more salts of iodide.
6. The method of claim 4, which further comprises contacting the spores with a surfactant.
7. The method of any of claims 4-6, wherein the spores are located on a surface.
- 20 8. The method of claim 7, wherein the surface is a textile surface.
9. The method of claim 7, wherein the surface is a surface of laboratory or process equipment.
- 25 10. A method of decontaminating a location, which has been exposed to spores, comprising contacting the spores with a laccase or a compound exhibiting laccase activity, a source of oxygen, a source of iodide ions and an enhancing agent.
11. The method of claim 10, wherein the source of iodide ions is one or more salts of iodide.
- 30 12. The method of claim 10, which further comprises contacting the spores with a surfactant.
13. A container comprising the composition of any of claims 1-3, wherein the components of the composition are packaged in one or more compartments or layers.
- 35 14. A ready-to-use sporocidal formulation comprising the composition of any of claims 1-3.
15. Use of a laccase for killing spores.

**ABSTRACT**

The invention provides a sporocidal composition comprising a laccase or a compound exhibiting laccase activity, a source of oxygen, a source of iodide ions and an enhancing agent.

- 5 A method of killing or inactivating spores and a method of decontaminating a location, which has been exposed to spores, are also disclosed.